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Rapid Conformational Analysis of Protein Drugs in Formulation by Hydrogen/Deuterium Exchange Mass Spectrometry

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ABSTRACT

Hydrogen deuterium exchange coupled to mass spectrometry (HDX-MS) has become an established method for analysis of protein higher order structure. Here, we use HDX-MS methodology based on manual solid-phase extraction (SPE) to allow fast and simplified conformational analysis of proteins under pharmaceutically relevant formulation conditions. Of significant practical utility, the methodology allows global HDX-MS analyses to be performed without refrigeration or external cooling of the setup. In mode 1, we used dimethyl sulphoxide–containing solvents for SPE, allowing the HDX-MS analysis to be performed at acceptable back-exchange levels (<30%) without the need for cooling any components of the setup. In mode 2, SPE and chromatography were performed using fast isocratic elution at 0°C resulting in a back-exchange of 10%-30%. Real-world applicability was demonstrated by HDX-MS analyses of interferon-β-1a in formulation, using an internal HDX reference peptide (P7I) to control for any sample-to-sample variations in back-exchange. Advantages of the methodology include low sample use, optimized excipient removal using multiple solvents, and fast data acquisition. Our results indicate that HDX-MS can provide a reliable approach for fast conformation analysis of proteins in their intended formulations, which could facilitate an increased use of the technique in pharmaceutical development research.

Introduction

Hydrogen deuterium exchange coupled to mass spectrometry (HDX-MS) is a well-established method for investigating higher order structure (HOS) and dynamics of proteins and is increasingly used in comparative studies on protein drugs (biologics) and their biosimilar candidates.[1,2] HDX-MS monitors the hydrogen and deuterium exchange reaction of backbone amide hydrogens when a protein is diluted into deuterated (D_2O) buffer. The shift in mass as a function of time due to uptake of the heavier deuterium isotope forms a unique pattern for each protein (or its peptide fragments) and can be used as a fingerprint to interpret its HOS and dynamics.[3,4] The main advantages of HDX for conformational characterization of proteins include high sensitivity, low sample requirement, the ability to characterize large proteins including mAbs, the ability to investigate the solution-phase HOS and dynamics of the protein,[5,6] and the possibility to pinpoint and localize changes to these at the residue level.[7]

On account of the rapid growth in development and use of biologic drugs,[8-10] the application of HDX-MS in characterization of HOS and dynamics of protein drugs is expanding. High-resolution methods for HOS characterization of proteins (such as nuclear magnetic resonance [NMR] and X-ray crystallography) are often not applicable in a high-throughput manner, and their utility in the analysis of large and complex biologic molecules such as mAbs is often limited.[11] In contrast, HDX-MS has been successfully used to characterize a variety of biologic drugs such as interferons[12] and a number of mAbs.[13-15] However, one major challenge to the use of higher resolution methods such as NMR, X-ray, and HDX-MS, in protein drug applications, is low tolerance of such methods to the presence of formulation excipients that interfere with analysis (salts, detergents, etc.).

Excipients are additives to the formulation matrix which serve, for example, to stabilize the protein drug. They ensure optimal shelf life and physiological osmolality in the final formulation. Most analytical methods for HOS characterization require excipient removal before analysis. However, this extra purification step may compromise and affect the structural integrity of the isolated protein, particularly with respect to HOS.[11,16,17] Moreover, most

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conventional methods in use for HOS characterization of protein therapeutics (CD, NMR, FT-IR, and X-ray) require conditions that are not representative of the original protein solution, and thus, obtained results do not accurately reflect any beneficial effects of formulation excipients on the protein HOS. Therefore, novel methods that allow HOS characterization of protein drugs in the presence of formulation excipients will be of great value in future drug development.

HDX-MS methodology has been applied to study the effect of formulation conditions on HOS of biologics.[18-21] However, routine application of HDX-MS to protein therapeutics in formulation is limited by the presence of excipients that have detrimental effects on both liquid chromatography (LC) (e.g., deteriorating separation performance in the case of detergents) and mass spectral quality (e.g., suppressing ionization). The removal of such excipients by complex and lengthy sample treatment steps at nonquench conditions is not feasible due to the concomitantly accelerated loss of deuterium label due to inevitable exposure and exchange of the deuterium in the protein with hydrogen atoms present in solvents used during the workflow. This phenomenon is also known as "back-exchange" and should be assessed and minimized in every new HDX-MS method. High levels of back-exchange result in loss of sensitivity of the method to detect differences in HDX between protein states and furthermore in false identification of the isotope exchange fingerprint of the protein or its peptides. Although analysis time, pH, and temperature have the largest effect on the back-exchange observed, other parameters such as protein sequence, ionic strength, solvent, and pressure can also affect back-exchange.[22,23] In a typical bottom-up HDX-MS workflow, back-exchange can range from 10%-50%, depending on the experimental setup and the protein studied.[24] Back-exchange values will increase further in the case of sample cleanup time in excess of 5-10 min even if quench conditions are maintained. Some earlier studies have aimed at improving HDX workflows by reducing back-exchange.[25,26] Furthermore, rapid separation systems for sample cleanup and desalting before MS have been reported.[27-29] Currently, however, at least in part due to challenges listed previously, HDX-MS is not often used routinely in drug development. For such later stage pharmaceutical applications, a tailored HDX-MS method capable of rapid quality control and screening of proteins with regards to their conformational stability in complex matrices (especially therapeutic proteins in pharmaceutical formulation) would be highly useful.

Here, we describe the optimization and application of a simple and inexpensive solid-phase extraction−HDX-MS setup (SPE-HDX-MS) capable of efficient removal of interfering formulation excipients during global HDX-MS analysis of protein pharmaceuticals. The method enables minimum sample preparation and fast data acquisition and bypasses purification steps that might affect the HOS of the protein. Furthermore, we show that the HDX-MS experiment can be carried out at room temperature without compromising back-exchange. The setup should be particularly useful for conformational analyses during rapid preformulation screening of protein drugs in different formulations.

Experimental

Chemicals, Reagents, and Materials

All solutions used were of HPLC grade. Acetonitrile, formic acid (FA), methanol (MeOH), dimethyl formamide (DMF), dimethyl sulphoxide (DMSO), guanidine hydrochloride (GnHCl), D_2O, tris (2-carboxyethyl) phosphine hydrochloride (TCEP), and polysorbate 20 were purchased from Sigma-Aldrich (St. Louis, MO). MilliQ water was obtained from Direct-Q® 3UV purification system (EMD Millipore, Billerica, MA) and used through the entire experiment. Unless mentioned otherwise, the mobile phase used throughout the entire experiment consisted of 0.23% FA in water as aqueous solvent (referred to as "*Solvent A*") and 0.23% FA in acetonitrile as organic solvent (referred to as "*Solvent B*").

Angiotensin II (ATII, human) and insulin (INS, human, recombinant) were purchased from Sigma-Aldrich and used as test compounds. Interferon-β-1a (IFN, recombinant human, produced in CHO cells, 13.5 μM, pH 4.8) in formulation was provided by Biogen and used as a representative biologic drug in an optimized formulation buffer. A synthetic octapeptide with sequence of PPPPPPPI (here referred to as P7I) was purchased (GenScript, Piscataway Township, NJ) and used as an internal HDX reference peptide.

Sample Preparation and HDX Reactions

Throughout the entire experiment, a tris buffer solution (20 mM tris, pH 7.34), was used as "*Labeling buffer*" for preparation of nonlabeled control samples. The identical buffer was prepared with D_2O instead of water and used for preparation of labeled samples.

Back-Exchange Study on INS and ATII

To prepare equilibrium-labeled and nonlabeled control samples of ATII and INS, 180 pmol of ATII stock solution (5 μM, in 0.1% trifluoroacetic acid) and 600 pmol of INS stock solution (172 μM, in 67 mM phosphate buffer, pH 7.4) was mixed and diluted 1:9 (v/v) in deuterated "*Labeling buffer*." Nonlabeled control samples were prepared in the same way but were diluted with nondeuterated buffer. Both equilibrium-labeled and nonlabeled samples were incubated at room temperature (RT, 25°C) for 17 h. The HDX reaction was then quenched on ice by diluting 1:1 (v/v) each of the labeled and nonlabeled samples with phosphate buffer (300 mM, pH 2.30). The final pH of the solution was 2.5. Samples were immediately stored at −80°C until the time of analysis. The initial concentrations were calculated so that the final amount of each analyte injected onto the column after the 10-fold dilution in "*Labeling buffer*" and the 1:1 (v/v) addition of quench buffer was 22.8 pmol ATII and 75.9 pmol INS. The aforementioned experiments were performed in triplicates.

Global HDX Analysis of IFN in Formulation

A mix solution of IFN (11.7 μM) and P7I (13.0 μM) was prepared by adding P7I stock solution (lyophilized powder reconstituted in water to 100 μM) to IFN formulation (13.5 μM, pH 4.8). The concentrations were designed such that the final amount of P7I and IFN injected onto the column after the 10-fold dilution in "*Labeling buffer*" and the 1:1 (v/v) addition of quench buffer was 135 pmol IFN and 150 pmol P7I. This stock solution was used for HDX experiment of "*Pharmaceutical IFN*" and "*GnHCl-treated IFN*".

Heat and TCEP-treated IFN samples were prepared by addition of TCEP to IFN stock solution (13.5 μM, pH 4.8) to the final concentration of 10 mM, and incubation at 60°C for 60 min.

Global HDX experiment was performed on 3 preparations of IFN: (1) "*Pharmaceutical IFN*," which contained interferon-β-1a in its commercial formulation buffer; (2) The same preparation, to which a denaturant (6 M GnHCl) was added (referred to as "*GnHCl-treated IFN*", the pH of this solution was adjusted to match the pH of the "*Pharmaceutical IFN*" solution); (3) The same preparation of IFN that was incubated at 60°C for 60 min (we call it "*Heat and TCEP-treated IFN*"). HDX reaction on these samples was initiated with 10-fold dilution of IFN preparation in deuterated "*Labeling buffer*" and incubation at RT for 15 s, 1 h, and 40 h time points. The HDX reaction was then quenched by 1:1 (v/v) addition of ice-cold phosphate buffer (300 mM, pH 2.30), mixing, and immediate storage at −80°C. For HDX on "*GnHCl-treated IFN*" samples, the